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Why do proteases mess up with antigen presentation by re-shuffling antigen sequences?

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The sequence of a large number of MHC-presented epitopes is not present as such in the original antigen because it has been re-shuffled by the proteasome or other proteases. Why do proteases throw a spanner in the works of our model of antigen tagging and immune recognition? We describe in this review what we know about the immunological relevance of post-translationally spliced epitopes and why proteases seem to have a second (dark) personality, which is keen to create new peptide bonds.

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Introduction

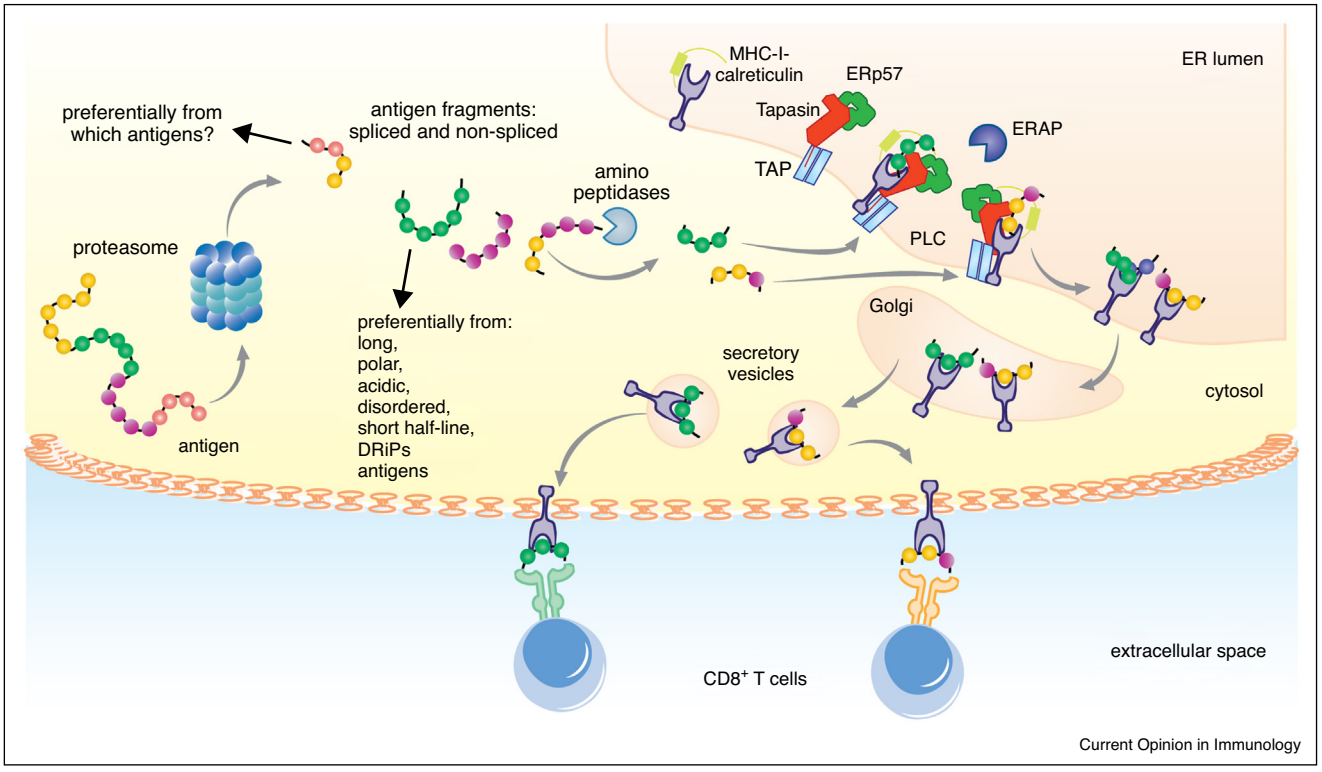
Epitopes can have their sequence re-shuffled by proteases, post-translationally modified, trimmed and bended onto MHC class I (MHC-I) molecules. Transformations can be so disguising that antigens might have trouble even recognizing themselves due to these non-canonical peptides. Nonetheless, the immune system seems to be able to selectively identify them non-canonical epitopes and use them for patrolling the status of the cell [1,2].

A growing number of studies about non-canonical epitopes has in part whipped out what we learned from textbooks about antigen presentation. For instance, intrinsic characteristics of non-canonical epitopes, especially of those derived from peptide splicing, force the boundaries of our conceptualization of the immunological self [3]. For example, a pre-requisite for streamlined CD8$^+$ T cells patrolling by recognizing antigenic spliced peptides is that their generation is tightly regulated. Indeed, if an arbitrary peptide fragment were ligated to another fragment we would likely have dramatic problems during thymocyte selection in the thymus due to an immense variety of spliced peptides presented by cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) and other medullary professional antigen presenting cells (APCs). According to the thymic selection models [4], only a handful of thymocytes would survive the negative selection with such an immense antigenic peptides' variety presented by professional APCs. In agreement with the pre-requisite for streamlined patrolling by CD8$^+$ T cells, there is a growing body of evidence that peptide splicing — and in particular proteasome-catalyzed peptide splicing (PCPS) — is not a random process, and only a minor portion of the theoretical spliced peptide is generated and presented to T cells. What these driving forces are, and implications they can have on the immune response is still to be fully understood.

The MHC-I antigen presentation pathway is on the contrary well described (Figure 1). CD8$^+$ T cell activity is strongly regulated by which epitopes are presented onto MHC-I complexes, that is, the MHC-I immunopeptidome. Alterations of the MHC-I immunopeptidome affect the cytotoxic CD8$^+$ T cell response against viruses and the efficacy of anti-cancer immunotherapies [5••,6,7]. The immunopeptidome is influenced by several factors (see Figure 1) including antigen availability and characteristics [8,9], proteasome processing [1], transport into the endoplasmic reticulum (ER) and loading to the peptide loading complex (PLC) [10••], trimming by ER amino peptidases (ERAPs) [7,11], as well as affinity to different MHC-I allotypes' clefts [12].

Part of the MHC-I immunopeptidome can derive from non-canonical reading frames [13], polymorphic or mutated sequences [2,14,15], non-coding sequences and DRiPs [16,17], or post-translationally modified peptides [18,19]. The predominant non-canonical peptide population seems to be, however, represented by spliced peptides. Indeed, in the MHC-I immunopeptidomes of human EBV-immortalized B cells and primary fibroblasts around 20–30% of peptides are produced by PCPS [20••]. Although the average number of molecules of spliced peptides bound to MHC-I complexes is smaller than that of non-spliced peptides [20••], spliced epitopes can be presented onto MHC-I complexes in the amount

Figure 1



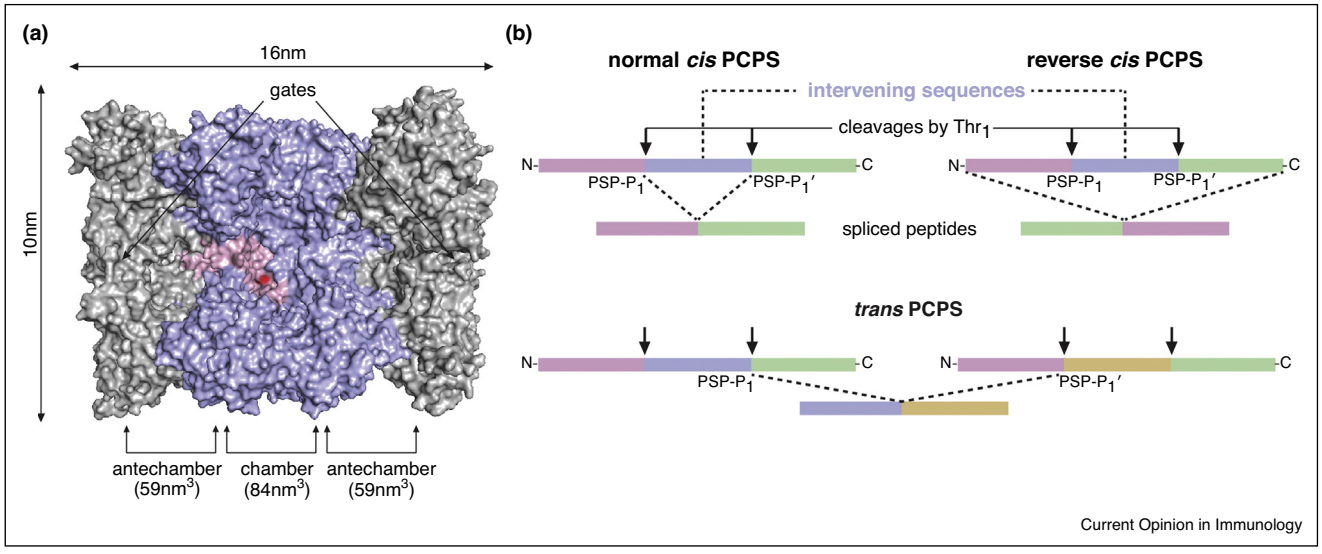
Antigen processing and presentation by MHC-I complexes to CD8$^+$ T cells. In this pathway, the majority of the antigens are processed by proteasome, which produces spliced and non-spliced peptides in the cytosol. Peptides are further degraded by amino-peptidases, thereby regenerating the cellular amino acid pool. Few peptides, however, are transported into the endoplasmic reticulum (ER) through the transporters associated with antigen processing (TAPs), which is part of the peptide-loading complex (PLC). There, peptides can be trimmed by ER-resident aminopeptidases (ERAPs). MHC-I-peptide complexes undergo modifications, and are transported through the Golgi to the cell surface. There, they can be recognized by the T cell receptor (TCR), and induce CD8$^+$ T cells priming/activation.

comparable to non-spliced epitopes [21]. For example, MHC-I-bound spliced epitopes have been found to prime a specific CD8$^+$ T cell response during *Listeria monocytogenes* infection [22$^•$]. Furthermore, a specific activation of CD8$^+$ T cells toward spliced epitopes derived from tumor-associated antigens is detectable in melanoma patients [21] and has led to a regression of the tumor mass in a melanoma patient and a leukemia murine model [23,24]. As a consequence, spliced peptides are interesting novel candidates for anti-viral vaccine development [25] and anti-cancer immunotherapies [1,26].

The molecular base of the double life of proteasome (and other proteases?) in permanent balance between cleavages and ligations

How does it come, however, that the proteasome and possibly other proteases in human cells seem to break and build again peptides so efficiently? We already know that PCPS efficiency is preserved along evolution [27] and that there are factors that promote PCPS. For instance, *in vitro* assays suggest that the proteasome favors the ligation of some peptide motifs [27,28], and where the proteasome prefers to cleave, it does not often splice [27]. However, to understand why the proteasome catalyzes so often PCPS, we need some information about its structure. The proteasome core particle is a barrel-shaped multi-subunit complex. In its internal cavity, it can accumulate up to 200–300 small peptides [29], or 2–3 proteins [30,31]. It has three pairs of catalytic subunits (β1, β2, and β5). The active site's threonine nucleophiles face the proteasome internal channel and are surrounded by the non-primed and primed substrate-binding sites (Figure 2a). The substrate degradation rate is driven by the proteolytic-site activity as well as by the peptide transport along the internal channel [29]. The catalytic subunit substitution, which is the hallmark differentiating proteasome isoforms, affects — at least at a quantitative level — cleavage-site preferences and the substrate degradation rate [29,32,33]. It also impacts PCPS, although, likely, only in a substrate-specific manner [21,27,34]. In cells, PCPS can occur via either transpeptidation or condensation [21,35], although the frequency of the latter mechanism still needs to be assessed. In *in vitro* assays, PCPS can efficiently occur by splicing fragments derived from the same molecule — *cis* PCPS — and from different molecules — *trans* PCPS —

Figure 2



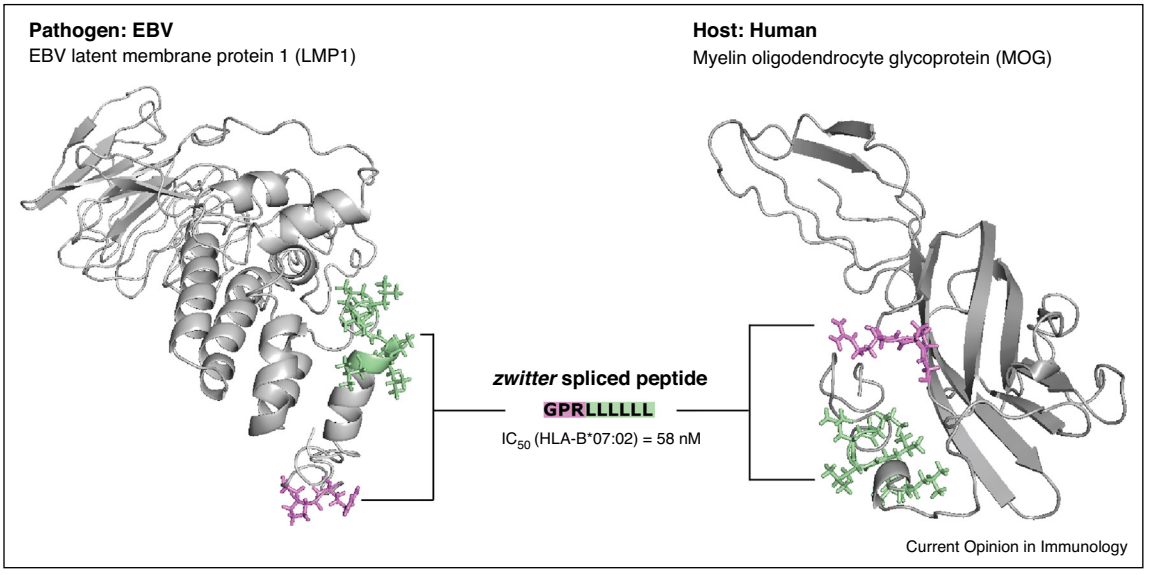
Molecular base for the unexpected high frequency of peptide splicing. **(a)** The human 20S core particle of the proteasome is shown based on the structure generated by [45]. The chains B, C, H, I, J, Q, R, S, Y and Z are hidden from the structure in order to see the inner proteasome cavities with the central chamber and its two antechambers. The α and β subunits are colored in grey and blue, respectively. As an example of a catalytic subunit, he β2 subunit is shown in pink with its active site threonine in red. **(b)** Proteasome-generated spliced peptides can be formed by: firstly, *cis* PCPS, when the two splice-reactants derive from the same polypeptide molecule; the ligation can occur in normal order, that is, following the orientation from N-terminus to C-terminus of the parental protein (normal *cis* PCPS), or in the reverse order (reverse *cis* PCPS); secondly, *trans* PCPS, when the two splice-reactants originate from two distinct protein molecules or two distinct proteins.

(Figure 2b) [27,28,36]. According to the transpeptidation model [35], the proteasome's catalytic N-terminal threonine nucleophile breaks the peptide bond of the residue (PSP-P1) of the protein — thereby forming an acyl-enzyme intermediate with the N-terminal splice-reactant, coupled to the release of the intervening sequence — and, instead of catalyzing the canonical peptide hydrolysis, it catalyzes the ligation between the PSP-P1 residue of the N-terminal splice-reactant with the residue PSP-P1′ of the C-terminal splice-reactant (Figure 2b). Proteasome-mediated transpeptidation can also result in isopeptide bond formation when a lysine side chain reacts with an acyl enzyme intermediate. Although it has also been shown that such peptides can induce an immune response, this process however remains to be observed *in vivo* [37].

Proteasome-generated sliced epitopes are however not the only examples of spliced epitopes. Indeed, Delong and colleagues [38••] identified some hybrid insulin peptides (HIPs), derived from the ligation of a fragment of proinsulin with peptides originating from other antigens present in the granules of the pancreatic β cells. These *trans* spliced epitopes are presented by MHC-II complexes, thereby triggering a specific response in $CD4^+$ T cells in type 1 diabetic patients [38••,39]. In general, extracellular antigens can be internalized, processed by proteolysis in the lysosome, bind the MHC class II molecules, and then circulate to the cell surface and back to the lysosome [40]. Although the lysosomal proteases also rely on (thio)ester intermediates, the enzyme catalyzing the production of the HIPs is still unknown.

Why do proteases (frequently) behave as ligases, too? In principle, any protease that uses a nucleophile to promote hydrolysis through an ester intermediate can catalyze transpeptidation. Hence any protease could play a role in the formation of spliced peptides. Although it is not understood why the proteasome in particular seems to catalyze this process so efficiently, we can speculate on the reasons. Transpeptidation efficiency depends highly on three factors: firstly, high concentration of the amine nucleophile must be present in order to favor the formation of a novel peptide-bond over hydrolysis; secondly, the ester needs to have a sufficient lifetime in order to allow the reaction and peptide bond formation over hydrolysis; thirdly, the active site in which this ester intermediate is formed must be sufficiently accessible for nucleophiles to react. The proteasome structure can favor all these three conditions, as it's a closed barrel that could have a high local concentration of peptide products, and use substrate-binding sites in proximity to the proteasome's catalytic N-terminal threonine nucleophile. Another result of peptides confinement in the proteasome barrel could be the fact that *trans* PCPS seems to occur less frequently than *cis* PCPS [27,28,36].

Figure 3



Example of *zwitter* peptide potentially generated from both EBV and myelin antigens. The theoretical *zwitter* peptide [GPR][LLLLLL] can be generated from both, the EBV antigen LMP1 and the human myelin protein MOG, through *cis* peptide splicing. This *zwitter* peptide is predicted to bind the HLA-B*07:02 variant with an IC_{50} of 58 nM, and it is one of the 13 peptides that are predicted to strongly bind to the most common MHC-I molecules. In this analysis the binding affinity is predicted applying the SMM prediction method [46], filtered for peptides with rank ≤ 1. The 13 theoretical *zwitter* peptides are predicted to bind one of the following variants: HLA-A*01:01, HLA-A*02:01, HLA-B*07:02, HLA-B*08:01, or HLA-B*40:01 (data not shown).

Theoretical impact of PCPS in recognizing the immunological self

One major feature of peptide splicing is the theoretical increase of the number of sequences that can be derived from the antigen pool and be allocated in the MHC-I and MHC-II clefts. This enlargement could have implications in the recognition of the immunological self by T cells. Indeed, it could increase the risk of mimicry, which is the phenomenon whereby two epitopes have sequence similarities and are recognized by the same T cell clone [1]. In particular, we name as '*zwitter* peptide' a peptide that can be derived from the human self-proteome as well as from a pathogen proteome (Figure 3). In 2012, Calis *et al.* [41] investigated the sequence overlap between human self-peptides and a large set of non-self-peptides derived from viruses and bacteria in the context of $CD8^+$ T cell recognition. They found that less than 1% of all theoretical possible 9-mer non-spliced peptides derived from pathogens have a sequence identical to the theoretical human non-spliced peptides, that is, are *zwitter* peptides. If the *zwitter* antigenic peptides were presented similarly by mTECs and other professional APCs in the medullary thymus and by dendritic cells (DCs) in the periphery, we would expect the absence, at the periphery, of $CD8^+$ T-cell clones recognizing, with high affinity, the *zwitter* peptides presented by DCs, because they have been eliminated during the thymic negative selection [4]. This phenomenon could in part explain the occurrence of holes in the T cell repertoire and in their ability to tackle viral infections [41]. On the contrary, if the *zwitter* antigenic peptides were efficiently presented by DCs and other APCs in the periphery but not by mTECs and other professional APCs in the thymic medulla, we would expect at the periphery the presence of potentially autoreactive $CD8^+$ T-cell clones, which could be primed and activated by DCs and other APCs in lymph nodes during the pathogen infection and afterwards attack human cells and participate to in an autoimmune response.

In multiple sclerosis, for instance, myelin-reactive $CD8^+$ T cell are theorized to mediate the cytotoxic activity against the oligodendrocytes leading to the characteristic de-myelination and plaque formation. Furthermore, associations between multiple sclerosis, some MHC-I variants (e.g. HLA-B*07) and Epstein-Barr virus (EBV) infection have been reported, and it has been hypothesized that an EBV infection could trigger the priming of autoreactive $CD8^+$ T cell clones through mimicry [42]. Using a similar approach as Calis *et al.* [41], we can compare the overlap of theoretical 9mer peptides (either spliced or non-spliced) derived from 24 human myelin proteins (MBP, MAG, MOG, PLP and isoforms) and from 9 EBV antigens (i.e. LMP1, LMP2, BMLF1, BMRF1, BZLF1, BRLF1, BNRF1, BLLF1, EBNA3). All *27* peptides theoretically common to myelin and EBV antigens are spliced peptides, since there are no identical non-spliced peptide sequences between these two sets of antigens. Among the *27* theoretical *zwitter* peptides, 13 peptides are predicted

to strongly bind to common MHC-I variants (see also Figure 3). Of course, this preliminary computation accounts for only the theoretical presence or absence of a peptide in the immunopeptidome of APCs. To better estimate the real prevalence of *zwitter* antigenic peptides and their recognition by $CD8^+$ T cells, we should consider the TCR degeneracy, the affinity/avidity of TCRs and MHC-I-peptides, and the dynamics of the different steps of the MHC-I antigen presentation (Figure 1) including the, only partially described, driving forces of PCPS. This preliminary *in silico* result, however, confirms that PCPS could play a particularly relevant role in the central tolerance, the occurrence of large holes in the T cell repertoire, and the autoimmune response mediated by $CD8^+$ T cells.

Concluding remarks

The surprising evidences reported in the last years, which suggest that MHC-I (and in part MHC-II) immunopeptidomes are populated by spliced peptides, need to be confirmed by applying different approaches before understanding the magnitude of their immunological relevance. However, the implications of peptide splicing could exceed the edges of antigen presentation. If peptide splicing were a common reaction for other proteases rather than proteasome, we could speculate that post-translationally spliced peptides (and why not spliced proteins?) could be involved in other aspects of the immune response and cell metabolism, as it has been proved for proteasome-processed non-spliced peptides and proteins [43,44]. If this hypothesis were correct, peptide splicing could be a further regulatory layer in the life of a cell and an organism.

Conflict of interest statement

Nothing declared.

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